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<b>(54) Title:</b> USE OF HISTONE DEACETYLASE INHIBITORS TO ACTIVATE TRANSGENE EXPRESSION  <b>(57) Abstract</b>  The invention provides methods for activating transgene expression by administering histone deacetylase inhibitors, methods for identifying compounds that activate transgene expression, and cells that can be used in these screening methods.		

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USE OF HISTONE DEACETYLASE INHIBITORS  
TO ACTIVATE TRANSGENE EXPRESSION

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Background of the Invention

This invention relates to methods for activating transgene expression, as well as methods for identifying compounds that can be used to activate transgene expression.

10        Treatment of genetic diseases will ultimately rely on gene therapy methods, in which new genes are delivered into patients' cells to make up for the presence of defective genes in the cells. One common problem in gene therapy is that expression of delivered genes is often completely shut down or dramatically decreased, particularly as time passes after gene delivery. For example, in  
15        transgenic mice, expression of a  $\beta$ -globin gene, that was introduced into the genomes of the mice in a retroviral vector, has been observed to be shut down by retroviral long terminal repeats (LTRs) (McCune et al., Nucl. Acids Res. 22(21):4477, 1994). Similar to retroviral LTRs, adeno-associated virus (AAV) inverted terminal repeats (ITRs) significantly down-regulate expression of  
20        transferred genes in mice, although to a lesser extent than retroviral LTRs.

Summary of the Invention

The invention is based on the observation that treatment of cells containing integrated transgenes with histone deacetylase inhibitors, such as butyrate and trichostatin A, activates expression of the transgenes.

25        Accordingly, the invention provides methods for increasing expression of transgenes, such as integrated transgenes, in cells. In these methods, cells are contacted with a histone deacetylase inhibitor, e.g., a histone H3 or H4 deacetylase

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(e.g., HDAC-1 and HDAC-2) inhibitor, such as butyrate (e.g., an aliphatic butyrate salt, sodium phenyl butyrate, sodium 4-phenyl butyrate, sodium butyrate, or arginine butyrate), trichostatin A, or trapoxin. These methods can be used to increase the expression of transgenes in any cell type into which transgenes can be introduced. For example, the methods can be used to increase expression of transgenes in hematopoietic cells, e.g., erythrocyte precursor cells, skin cells, such as keratinocytes, as well as liver, lung, and brain cells. The transgene may have been introduced into the cell, or a precursor of the cell, using a vector that facilitates integration, such as an adeno-associated viral (AAV) vector, a retroviral vector (e.g., a Moloney Murine Leukemia viral vector), or by a vector that may or may not cause integration of the transgene, such as a human or bovine papilloma viral vector. Alternatively, as is described further below, any of a number of well known transfection methods, such as calcium phosphate precipitation, electroporation, microinjection, and liposome-mediated transfection, can be used.

Also included in the invention is the use of a histone deacetylase inhibitor, for example, a histone H3 or H4 inhibitor, such as a derivative of butyrate (e.g., sodium butyrate), trichostatin A, or trapoxin, in the preparation of a medicament for increasing expression of a transgene in a cell.

The invention also includes methods for identifying activators of transgene (e.g., integrated transgene) expression in a cell. In these methods, a cell (e.g., a HeLa or K562 cell) containing a transgene (e.g., the *lacZ* gene) is provided and cultured in the presence of a candidate compound (e.g., a histone deacetylase inhibitor, such as a derivative of butyrate, trichostatin A, or trapoxin). Expression of the transgene in the cell is monitored, and detection of an increased level of expression of the transgene in the cell cultured in the presence of the candidate compound, compared to the level of expression of the transgene in a control cell cultured in the absence of the candidate compound, indicates that the candidate compound is an activator of transgene expression. As is described further below,

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the transgene can have been introduced into the cell by any standard method. For example, the transgene can have been introduced into the cell using a viral vector (e.g., an adeno-associated or retroviral vector).

5 In addition, the transgene in such a cell can be operably linked to a promoter, such as the Cytomegalovirus promoter. By "operably linked" is meant that a gene and a regulatory sequence(s), such as a promoter, are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins or proteins which include transcriptional activation domains) are bound to the regulatory sequence(s).

10 The invention also includes a cell (e.g., a HeLa or K562 cell) containing an integrated transgene (e.g., the *lacZ* gene) that was introduced into the cell using an adeno-associated viral vector, in the absence of a selectable marker. The transgene can be operably linked to a promoter, such as the Cytomegalovirus promoter.

15 The invention provides several advantages. For example, the treatment methods of the invention facilitate prolonged expression of therapeutic transgenes, which can minimize the need for repetition of gene therapy treatments. The screening methods of the invention can be used to identify additional compounds for use in the treatment methods of the invention. For example, compounds that are characterized by increased efficacy or safety can be identified.

20 Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

#### Brief Description of the Drawings

25 Fig. 1A is a graph showing the relative  $\beta$ -galactosidase activity levels in FACS sorted pool 4 cells (with hydroxyurea treatment) that were treated with butyrate or trichostatin A, or left untreated.

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Fig. 1B is a graph showing the relative  $\beta$ -galactosidase activity levels in FACS sorted pool 2 cells (without hydroxyurea treatment) that were treated with butyrate or trichostatin A, or left untreated.

Figs. 2A and 2B are photographs of Southern blot analyses of HeLa cell clones after rAAV/CMV*lacZ* infection. HeLa cells were infected with rAAV/CMV*lacZ*, grown for 3 weeks, and then plated at limiting dilution. Individual cells were expanded for 4 weeks, and DNA was extracted for analysis. Genomic DNA from uninfected HeLa cells was used as a negative control, and a 5.0 kilobase fragment from the rAAV/CMV*lacZ* vector was spiked into HeLa cell DNA for a positive control. A 3.0 kilobase *lacZ* DNA fragment was used as the probe. In Fig. 2A, the genomic DNA was digested with *ScaI*, which does not cut rAAV/CMV*lacZ*. In Fig. 2B, the genomic DNA was digested with *XbaI*, which cuts once inside the vector.

Fig. 2C is a schematic representation of the rAAV/CMV*lacZ* virus.

Fig. 3 is a photograph of control cells and cells treated with azacytidine, butyrate, or trichostatin A. These cells were stained with X-gal. The results for 5 typical clones are shown. Clones 2 and 135 represent a group of clones in which every cell turned blue after treatment with butyrate or trichostatin; clones 141 and 162 represent a group of clones in which a variable percentage (typically about 30-90%) of the cells turned blue with butyrate or trichostatin A treatment; and clone 64 represents a group of clones in which *lacZ* was constitutively expressed.

Figs. 4A and 4B are photographs of Southern blot analyses of rAAV/HS2 $\alpha\beta^{AS3}$ -transduced K562 cell clones. Cells were infected, grown for 30 days, and plated at limiting dilution. Individual cells were expanded for 4 weeks, and DNA was extracted and analyzed as described for the HeLa cell clones, except that genomic DNA from uninfected K562 cells was used as a negative control and a 4.5 kilobase fragment of the rAAV/HS2 $\alpha\beta^{AS3}$  vector spiked into K562 cell DNA

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was used for a positive control. The 790 basepair *HinfI-HinfI* fragment of the  $\beta$ -globin gene *IVS2* was used as a probe. In

Fig. 4A, genomic DNA was digested with *ScaI*, which does not cut in rAAV/HS2 $\alpha\beta^{AS3}$ . Hybridization of the probe to high molecular weight bands  
5 (greater than 25 kilobases) demonstrates that viral sequences are not present as episomal vectors. In Fig. 4B, genomic DNA was digested with *EcoRI*, which cuts once in the  $\beta$ -globin gene. The single junction fragments that hybridize to the probe demonstrate that all six clones contain single copy integrants of the transduced gene. The endogenous  $\beta$ -globin gene fragment (5.5 kilobase) ran  
10 slightly faster than expected in this gel.

Fig. 4C is a schematic representation of rAAV/HS2 $\alpha\beta^{AS3}$ .

Fig. 5 is a photograph of RT-PCR analyses of six representative K562 clones, each containing integrated rAAV/HS2 $\alpha\beta^{AS3}$ . K562 cell clones were treated with sodium butyrate (BU) or trichostatin (TSA) for 24 hours; untreated cells were  
15 included as controls (CTL). Total RNA was extracted with RNA STAT60. RT-PCR of endogenous  $\alpha$ -globin mRNA served as an internal control. A dramatic induction of  $\alpha\beta^{AS3}$  gene expression was observed in half of the clones. The level of butyrate induction was 8.1-fold for clone 237, 33-fold for clone 396, and 9-fold for clone 658. The level of trichostatin A induction was 10.2-fold for clone 237, 19-  
20 fold for clone 396, and 14-fold for clone 658. Overall,  $\alpha\beta^{AS3}$  expression was inducible in 6 of the 13 clones, and the average level of induction was 10.6-fold and 9.0-fold for butyrate and trichostatin A, respectively. Expression of  $\alpha\beta^{AS3}$  in the other half of the samples was constitutive as illustrated by clones 260, 354, and 394.

25 Fig. 6 is a schematic representation of a model for silencing and reactivation of recombinant viral genes. A host protein or protein complex binds to viral sequences (AAV ITRs or retroviral LTRs) and recruits a histone deacetylase to the site through protein-protein interactions. The enzyme deacetylates histone H3 and

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- H4 N-terminal tails, and the resulting change in chromatin structure inhibits expression from adjacent promoters. Treatment with trichostatin A specifically inhibits histone deacetylases. Subsequent acetylation of histones produces a chromatin structure that allows transcription factors to bind to nearby promoters and activate gene expression.

### Detailed Description

- The invention provides methods for increasing expression of transgenes, such as integrated transgenes, in cells by treating the cells with histone deacetylase inhibitors, such as sodium butyrate and trichostatin A. Also included in the invention are methods for identifying additional compounds that can be used to increase expression of transgenes (e.g., integrated transgenes), as well as cells that can be used in these screening methods. These methods and cells are further described as follows.

#### 15 *Use of histone deacetylase inhibitors to increase transgene expression*

- The invention provides methods for increasing transgene expression. In these methods, a patient that has been treated by gene therapy is administered a histone deacetylase inhibitor, e.g., a histone H3 or H4 deacetylase inhibitor, such as butyrate or trichostatin A. Treatment can be carried out concurrently with gene therapy or after the gene therapy has been completed, e.g., ten or more days later. In the case of gene therapy involving the *ex vivo* introduction of genes into, e.g., hematopoietic stem cells (see, e.g., Luhovy et al., *Biology of Blood and Marrow Transplantation* 2:24, 1996), the treatment with a histone deacetylase inhibitor is preferably commenced after the patient has recovered from the transplant.

- 25 Histone deacetylase inhibitors can be administered to patients using any standard method determined to be appropriate by one skilled in the art. For example, intravenous (see, e.g., Perrine et al., *New Eng. J. Med.* 328(2):81, 1993)



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or oral (see, e.g., Dover et al., Blood 84(1):339, 1994) administration can be used. In addition to these preferred routes of administration, any other appropriate route of administration can be employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intraperitoneal, or intranasal administration routes can be used. For parenteral administration, the therapeutic formulations can be in the form of liquid solutions or suspensions. For oral administration, the formulations can additionally be in the form of tablets or capsules, and for intranasal formulations, the formulations can additionally be in the form of powders, nasal drops, or aerosols. Methods for making such formulations can be found, for example, in "Remington's Pharmaceutical Sciences," which is a standard reference in the pharmaceutical field.

Appropriate dosage levels and administration schedules for histone deacetylase inhibitors can readily be determined by one skilled in the art. Butyrate, for example, can be administered in amounts ranging from 1-40 g/day, for example, 3.5-20 g/day. As a specific example, a daily dosage of 20 g sodium phenyl butyrate, in the form of multiple 0.5 g tablets, can be orally administered to adults with meals in three divided doses (see, e.g., Dover et al., *supra*). Alternatively, arginine butyrate, in amounts ranging from 200-3,000 mg/kg body weight, e.g., 1,000-2,000 mg/kg body weight, can be administered daily by continuous intravenous infusion (see, e.g., Perrine et al., *supra*). Trichostatin A can be administered to patients in amounts ranging from, e.g., 0.1-40 mg/day, such as 3.5-20 mg/day. Additional appropriate dosage ranges can readily be determined by one skilled in the art.

Treatment can be carried out in, e.g., daily, weekly, bi-monthly, monthly, or bi-yearly schedules, as determined to be appropriate by one skilled in the art. Factors influencing the frequency and duration of treatment include the level of expression of the delivered gene, the turn-over rate of the transduced cell type, as well as the detection of beneficial and/or adverse effects of the treatment in the

patient. Dosage levels and the frequency of administration can be altered as determined to be appropriate by one skilled in the art, e.g., by monitoring patients for drug side effects and for symptoms of the disease sought to be treated. In some cases, the level of expression of a gene introduced into a patient by gene therapy, e.g., a gene introduced into a hematopoietic stem cell, can be monitored directly, e.g., by blood sample analysis. In these cases, the levels of expression can be monitored to determine when adjustment of dosage levels and/or frequency of administration is appropriate.

In addition to the histone deacetylase inhibitors described above, the treatment methods of the invention can employ the use of additional compounds that activate transgene expression, which can be identified using the methods described below.

*Methods for identifying compounds that increase transgene expression*

Compounds that can be used to activate transgene (e.g., integrated transgene) expression in cells, such as histone deacetylase inhibitors in addition to those described above, can be identified using the screening methods that are described as follows. Preferably, a candidate compound (e.g., a derivative of a histone deacetylase inhibitor listed above) is first tested in a cell culture assay, and, if favorable results are obtained in the cell culture assay, the candidate compound is then tested in an animal model system.

In the cell culture assay, cells that have been cultured long enough so that expression of a transgene in the cells has been silenced, are cultured in the presence of a candidate compound, and the level of expression of the transgene in the cells is compared to the level in control cells that contain the transgene, but are cultured in the absence of the candidate compound. Detection of an increased level of transgene expression in the cells cultured in the presence of the candidate compound, compared to the control, indicates that the compound can be used in

methods to increase expression of transgenes, such as the methods described above.

Any cell that can be maintained in culture, and, optionally, into the genome of which a transgene can be integrated, can be used in the screening methods of the invention. For example, HeLa cells or K562 cells, which are described further  
5 below, as well as fibroblasts and keratinocytes, can be used in the screening methods. Cells that can be used in the screening methods of the invention can be obtained from numerous commercial sources, for example, the American Type Culture Collection (ATCC, Rockville, Maryland).

The level of expression of a transgene in a cell can be measured at the levels  
10 of transcribed RNA or translated protein using any of numerous methods known in the art. For example, the level of translated protein, either in intact cells or cell extracts, can be detected using immunological methods, such as immunofluorescence methods, ELISA, RIA, immunoprecipitation, and Western blot analysis. If the transgene encodes a protein having enzymatic activity, the  
15 level of translated protein can be monitored using assays that detect the specific enzymatic activity. Specific examples of reporter genes encoding enzymes that can be used in the invention include, for example, *lacZ* (see below), chloramphenicol acetyl transferase (CAT), luciferase, and green fluorescent protein (GFP) (see, e.g., Ausubel et al., eds. *Current Protocols in Molecular Biology*, Wiley & Sons, New  
20 York, 1989). The level of transcribed RNA can be detected directly using methods such as RT-PCR, which is described further below, as well as nuclease protection assays, Northern analysis, and *in situ* hybridization (see, e.g., Ausubel et al., *supra*).

A transgene can be introduced into cells for use in the screening assays of the invention using any of numerous well known methods. For example, a viral  
25 vector, such as an AAV vector (see below) or a retroviral vector (e.g., a Moloney Murine Leukemia viral vector), can be used. Alternatively, the transgene can be

introduced into cells using liposomes (e.g., by use of lipofectin) or by other standard transfection methods, such as calcium phosphate precipitation, electroporation, or microinjection (see, e.g., Ausubel et al., *supra*). Optionally, the transgenes can be integrated into the genomes of the cells. Finally, if a selectable  
5 marker is used, selection medium can be removed and the cells cultured for an additional amount of time to allow silencing.

Before introduction into the cells, the transgene generally is operably linked to a promoter that can direct expression of the transgene in the cells. Numerous promoters are well known, and readily obtainable, by those skilled in the art. For  
10 example, a non-tissue specific promoter, such as the Cytomegalovirus (CMV) promoter (DeBernardi et al., Proc. Natl. Acad. Sci. USA 88:9257-9261, 1991), can be used. Alternatively, a cell type-specific promoter can be used to direct expression in specific cells. For example, as is described further below, an  $\alpha$ -globin promoter can be used to direct transgene expression in erythroid cells, such  
15 as K562 cells.

Compounds that are found to increase transgene expression in the cell culture systems described above can be further tested in animal model systems. For example, animals (e.g., mice, rats, hamsters, rabbits, goats, and pigs) to which a gene therapy agent, such as a viral vector (e.g., a recombinant AAV vector)  
20 containing a transgene or a cell containing a transgene that was introduced into the cell by *ex vivo* gene therapy methods, has been administered can be monitored for transgene expression in the presence and absence of a candidate compound. Alternatively, transgenic animals can be used. Expression can be measured using methods that are appropriate for the tissue in which the expression is to be detected.  
25 For example, a sample of a tissue in which expression is being monitored can be taken by biopsy, or a physiological activity affected by expression of the transgene can be monitored.

The treatment and screening methods described above are based on the following experimental results and methods.

*Treatment with butyrate and trichostatin reactivates lacZ expression to a high and stable level in HeLa cells*

5           The expression of *lacZ* in HeLa cells infected with a recombinant AAV vector containing a Cytomegalovirus (CMV) promoter linked to the *E. coli lacZ* gene (rAAV/CMV*lacZ*) decreases over time to a very low level, and the level of *lacZ* can be dramatically enhanced or rescued by infection of the cells with wild type adenovirus. These results showed that down-regulated expression of  
10 rAAV/CMV*lacZ* can be rescued, and led to a search for drugs that can mimic the effect of adenovirus on transgene expression. As is described further below, we found that histone deacetylase inhibitors, such as sodium butyrate and trichostatin A, which are non-specific and specific histone deacetylase inhibitors, respectively, dramatically restored rAAV/CMV*lacZ* gene expression in stably transfected HeLa  
15 cells.

HeLa cells were treated with 4 mM hydroxyurea, which has been shown to increase AAV integration efficiency (Russell et al., Proc. Natl. Acad. Sci. USA 92:5719-5723, 1995), for 24 hours before infection with rAAV/CMV*lacZ*. After infection at a multiplicity of infection (moi) of 5, the cells were plated and  
20 examined for *lacZ* expression at 6, 10, 20, 30, 40, 50, and 60 days after infection. Hydroxyurea increased the number of blue cells in general (Tables 1 and 2), but it did not prevent expression of *lacZ* from decreasing over time. The number of hydroxyurea-treated, *lacZ*-expressing cells decreased progressively over time, until few cells expressed the transduced gene at day 60 (Table 1). The same silencing of  
25 *lacZ* expression was observed with cells that were not pretreated with hydroxyurea (Table 2). In an attempt to reactivate *lacZ* expression, the cells were treated with 5-azacytidine, which has been used to reactivate endogenous genes whose promoters

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or enhancers are methylated (Ferguson et al., Cancer Res. 55:2279-2283, 1995; Lee et al., Mol. Cell. Biol. 15:2547-2557, 1995; Yoshiura et al., Proc. Natl. Acad. Sci. USA 92:7416-7419, 1995), but 5-azacytidine did not reactivate *lacZ* expression (Tables 1 and 2), even when broad concentrations were used.

5 In contrast, treatment of the rAAV/CMV*lacZ*-infected HeLa cells with sodium butyrate dramatically reactivated *lacZ* expression. Even after 60 days, when *lacZ* expression was silenced in 99% of the cells, sodium butyrate treatment reactivated expression to high levels. Sodium butyrate inhibits histone deacetylases, but also has a number of other activities (Kruh, Mol. Cell. Biochem. 10 42:65-82, 1982). To determine whether the inhibition of histone deacetylation was the major cause of *lacZ* reactivation, rAAV/CMV*lacZ*-infected HeLa cells were treated with trichostatin A, which is a specific inhibitor of histone deacetylase (Yoshida et al., J. Biol. Chem. 265:17174-17179, 1990). The data in Tables 1 and 2 and Fig. 3 show that trichostatin A, like sodium butyrate, activates *lacZ* expression 15 to high levels. These data show that histone deacetylation is integrally involved in the silencing of transduced genes in this system, and that inhibition of histone deacetylases is a powerful method of reactivating gene expression.

**Table 1** Restoration of *lacZ* gene expression in hydroxyurea pretreated and rAAV/*lacZ* infected HeLa cells by post-treatment of cells with sodium butyrate or trichostatin A.

20	Control	Azacytidine	Butyrate	Trichostatin A	
	Day 6	447	375	994	1341
	Day 10	136	111	893	1168
	Day 20	130	136	1112	1428
	Day 30	37	50	795	1001
25	Day 40	34	26	835	1115
	Day 50	15	19	771	1076
	Day 60	23	24	934	1138

30 HeLa cells were pretreated with 4 mM hydroxyurea overnight, and then infected with rAAV/*lacZ* at MOI of 5 overnight. The infected cells were cultured and plated in 12 well plates at days shown in the table. After overnight plating, cells were treated with various drugs for 24 hours and then stained with X-gal. The numbers

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shown in the table represent the average blue cell number in each well without drug post-treatment (control), or with 3  $\mu$ M azacytidine, 50 mM sodium butyrate, or 3  $\mu$ M trichostatin A, post-treatment.

**Table 2** Restoration of *lacZ* gene expression in non-pretreated rAAV/*lacZ* infected HeLa cells by post-treatment of cells with sodium butyrate or trichostatin A.

5		Control	Azacytidine	Butyrate	Trichostatin A
10	Day 6	40	26	264	281
	Day 10	13	18	258	304
	Day 20	5	8	185	250
	Day 30	6	3	132	248
	Day 40	3	2	132	174
	Day 50	1	1	123	178
	Day 60	1	1	131	220

15 HeLa cells without pretreatment were infected with rAAV/*lacZ* at MOI of 5 overnight, then cells were treated in the same way as described in Table 1.

The effects of histone deacetylase inhibitors on *lacZ* expression in rAAV/CMV/*lacZ*-infected HeLa cells was further studied using fluorescence-activated cell sorting (FACS) analysis. As is described further below, at day 30 post-infection, HeLa cells infected with rAAV/CMV/*lacZ* were plated for 24 hours and then treated with 50 mM butyrate or 3  $\mu$ M trichostatin A for 24 hours. The cells were then treated with trypsin to form a single cell suspension, which was subjected to FACS analysis. When rAAV/CMV/*lacZ*-infected cells were FACS sorted twice, post-treatment with butyrate or trichostatin A dramatically increased *lacZ* expression. A quantitative assay of  $\beta$ -galactosidase activity demonstrated at least a 12 to 23-fold increase in enzyme activity after butyrate and trichostatin treatment (Figs. 1A and 1B). These values could be conservative, as FACS tended to sort cells constitutively expressing *lacZ* in control groups.

*All silenced integration sites were responsive to butyrate and trichostatin treatments*

Wild type AAV can integrate at a specific site on chromosome 19 (Kotin et al., Proc. Natl. Acad. Sci. USA 87:2211-2215, 1990; Samulski et al., EMBO J. 10:3941-3950, 1991), but recombinant AAV vectors that do not contain the *rep* gene normally integrate at random sites in the host genome (Walsh et al., Proc. Natl. Acad. Sci. USA 89:7257-7261, 1992). To determine the percentage of rAAV/CMV*lacZ* integration sites that are responsive to butyrate or trichostatin A treatment, HcLa cells were infected with rAAV/CMV*lacZ* (moi=1,000; Fig. 2C illustrates the rAAV/CMV*lacZ* construct) and cloned by limiting dilution. Stably transduced cells were identified by PCR and this identification was confirmed by Southern blot hybridization. Twenty-five clones were analyzed, and the Southern blot data for five representative clones are shown in Figs. 2A and 2B, which depict the results observed for *ScaI* and *XbaI* digestions, respectively. *ScaI* does not cut in rAAV/CMV*lacZ*. Thus, if the construct is integrated into the HeLa cell genome, a DNA fragment that is larger than unit length (5.0 kilobases) should hybridize with the *lacZ* probe. Fig. 2A shows that all five clones contain copies of rAAV/CMV*lacZ* in large *ScaI* fragments (greater than 20 kilobases). *XbaI* cuts rAAV/CMV*lacZ* once. Thus, digestion with this enzyme and hybridization with the *lacZ* probe should produce a junction fragment that is different for each integration site. A unit length fragment would be produced if head-to-tail copies are present. Fig. 2B shows that all five clones contain different rAAV/CMV*lacZ* junction fragments and that clones 141 and 162 also contain unit length fragments that hybridize with the probe. These results show that the clones contain stably integrated copies of rAAV/CMV*lacZ*.

Cells from each clone were assayed for *lacZ* expression with or without treatment with 5-azacytidine, sodium butyrate, or trichostatin A (Fig. 3). Few cells in four of the five untreated controls expressed *lacZ*, but both sodium butyrate and trichostatin A dramatically activated *lacZ* expression. Fig. 3 shows X-gal staining results of 5 typical clones, clones 2 and 135 representing a group of clones in which



every cell turned blue after treatment with butyrate or trichostatin, clones 141 and 162 representing a group of clones in which a variable percentage (typically about 30-90%) of cells turned blue with butyrate or trichostatin A treatment, and clone 64 representing a group of clones in which *lacZ* was constitutively expressed. In the total number of *lacZ* DNA positive clones, 80% were butyrate and trichostatin A responsive, and the remaining 20% were constitutively expressing  $\beta$ -galactosidase. As mentioned above, trichostatin A is a specific inhibitor of histone deacetylase. Thus, the hyperacetylation of histones that occurs in the presence of this drug appears to be responsible for *lacZ* reactivation. Interestingly, 1 of the 5 clones illustrated in Fig. 3, and 5 of the 25 clones overall, contained a constitutively active *lacZ* gene. Thus, 20% of the AAV integration sites analyzed in this study are not silenced in HeLa cells.

*Reactivation of gene expression by histone deacetylase inhibitors is not promoter or cell type-specific*

To determine whether reactivation of silenced, virally transduced genes by butyrate and trichostatin A is applicable to other promoters and cell types, a human  $\beta$ -globin gene vector was constructed. This vector contains the human  $\beta$ -globin locus control region DNase I Hypersensitive Site 2 (HS2), linked to a human  $\alpha$ -globin promoter driving an anti-sickling  $\beta$ -globin gene (see below). The human  $\beta$ -globin HS2 sequence was shown previously to direct high-level, erythroid-specific expression of  $\alpha$ - and  $\beta$ -globin genes in transgenic mice (Curtain et al., Proc. Natl. Acad. Sci. USA 86:7081-7086, 1989; Ryan et al., Genes Dev. 3:314-323, 1989; Ryan et al., Proc. Natl. Acad. Sci. USA 86:37-41, 1989; and Fraser et al., Nucleic Acids Res. 18:3503-3508, 1990) and cultured erythroid cells (Fraser et al., supra; Tuan et al., Proc. Natl. Acad. Sci. USA 86:2554-2558, 1989; Ney et al., Nucleic Acids Res. 18:6011-6017, 1990; and Ney et al., Genes Dev. 4:993-1006, 1990). The human  $\alpha$ -globin promoter was chosen because this promoter is less susceptible

to silencing induced by plasmid sequences (Townes et al., EMBO J. 4:1715-1723, 1985), retroviral LTR sequences (McCune et al., Nucleic Acids Res. 22:4477-4481, 1994), and AAV ITR sequences than the  $\beta$ -globin promoter. The  $\alpha$ -globin promoter has been used successfully by other investigators in AAV vectors (Ponnazhagen et al., J. Exp. Med. 179:733-738, 1994). Fig. 4C illustrates the rAAV/HS2 $\alpha\beta^{AS3}$  construct. Human erythroleukemia cells (K562) were infected with the virus, and 14 clones of stably transduced cells were obtained by limiting dilution and PCR analysis. Southern blots of six representative clones are illustrated in Figs. 4A and 4B. Digestion of genomic DNA with *ScaI*, which does not cut rAAV/HS2 $\alpha\beta^{AS3}$ , demonstrated that the viral sequences were incorporated into high molecular weight DNA (greater than 25 kilobases) and, therefore, were not present as episomal vectors (Fig. 4A). Digestion with *EcoRI*, which cuts only once in rAAV/HS2 $\alpha\beta^{AS3}$ , demonstrated single junction fragments indicative of single copy, random integrants (Fig. 4B). Head-to-tail tandem arrays would produce a unit length band of 4.5 kilobases, but this band was not observed. Thirteen of the 14 clones analyzed contained an intact copy of the transduced construct.

These 13 clones were tested for  $\alpha\beta^{AS3}$  expression by RT-PCR, which is described further below. The endogenous human  $\alpha$ -globin gene was chosen as an internal control because previous studies demonstrated that synthesis of the adult globins in K562 cells is not significantly induced or suppressed by butyrate treatment (Lozzio et al., Nature (London) 281:709-780, 1979; Cioe et al., Cancer Res. 41:237-243, 1981; and Guerrasio et al., Blood Cells 7:165-176, 1981). Fig. 5 illustrates RT-PCR analysis of the same six clones that were analyzed in Figs. 4A and 4B. In three of the clones, expression of  $\alpha\beta^{AS3}$  was dramatically induced by butyrate and trichostatin A. The level of butyrate induction was 8.1-fold for clone 237, 33-fold for clone 396, and 9-fold for clone 658. The level of trichostatin A induction was 10.2-fold for clone 237, 19-fold for clone 396, and 14-fold for clone

658. Overall,  $\alpha\beta^{AS3}$  expression was inducible in 6 of the 13 clones, and the average level of induction was 10.6-fold and 9.0-fold for butyrate and trichostatin A, respectively.  $\alpha\beta^{AS3}$  expression in the other seven clones was constitutive, and the data for three of these clones (260, 354, and 394) are shown in Fig. 5.

5        Fig. 6 illustrates a model for silencing virally transduced genes. A host protein or protein complex binds to viral sequences (AAV ITRs or retroviral LTRs) and recruits a histone deacetylase to the site through protein-protein interactions. The enzyme deacetylates histone H3 and H4 N-terminal tails in the region, and the resulting change in chromatin structure inhibits adjacent promoters. Treatment  
10        with trichostatin A specifically inhibits the deacetylase. Subsequent acetylation of histones produces a chromatin structure that allows transcription factors to bind to nearby promoters and activate gene expression.

The results set forth above were obtained using the following materials and methods.

#### 15        *Reagents and solutions*

1 M sodium butyrate (Sigma) was prepared in phosphate buffered saline (PBS); 1 mM trichostatin A (Wako Chemicals) was made in DMSO; 20 mM 5-azacytidine (Sigma) was prepared in 1:1 acetic acid:water; and 1 M hydroxyurea (Sigma) was dissolved in phosphate-buffered saline (PBS). All stock solutions were stored at -  
20        20°C, diluted to working concentrations in cell culture medium, and filter sterilized for tissue culture, except that 5-azacytidine-containing medium was adjusted to pH 7.3 before filtration. rAAV/CMV/lacZ viral lysates were prepared and titered as described (Luhovy et al., *Biology of Blood and Marrow Transplantation* 2:24, 1996). The titer of the viral lysate used in these studies was  $1 \times 10^8$  per mL. The  
25        lysate was heated at 56°C for 15 minutes to inactivate wild type adenovirus before being used for infection.

*HeLa cell infection and drug post-treatment*

HeLa cells were maintained in Dulbecco's Modified Eagles Medium (Mediatech), with 10% fetal bovine serum (Hyclone).  $2 \times 10^5$  cells were plated in each well of a 24-well plate overnight, then the medium was replaced with 1 mL  
5 fresh medium. Ten  $\mu$ L heat-inactivated viral lysate was then added per well and cultured for 24 hours. In the case of hydroxyurea pretreatment, overnight seeded HeLa cells were cultured in 1 mL medium containing 4 mM hydroxyurea for 24 hours. The medium was then aspirated, and the cells were rinsed twice with fresh medium. One mL fresh medium and 10  $\mu$ L viral lysate were added for  
10 infection, as described above. After 24 hours of infection, the cells were washed three times with PBS and fresh medium was added for culture. When confluency was reached, the cells were treated with trypsin and transferred to 6-well plates. When confluent, the cells were split and passaged. One-third of the cells were plated into a new 6-well plate, and the remaining cells were either frozen or plated  
15 into 12-well plates for drug treatment. The infected cells were cultured continually for up to 60 days.

At days 6, 10, 20, 30, 40, 50, and 60 post-infection,  $2 \times 10^5$  cells per well were plated into 12-well plates. After overnight seeding, the medium was replaced with fresh medium (control) or with medium containing 3  $\mu$ M 5-azacytidine, 50 mM  
20 sodium butyrate, or 3  $\mu$ M trichostatin A. After 24 hours post-treatment with these drugs, the cells were fixed and stained with X-gal using standard methods (see, e.g., Ausubel et al., *supra*). Blue cells were examined with an inverted microscope and counted.

*Sorting of rAAV/CMVlacZ-infected HeLa cells*

25 At day 30 post-infection, the infected cells were plated into a 100 mm plate overnight and then were treated with 50 mM butyrate or 3  $\mu$ M trichostatin A for 24 hours. Control cells were plated one day before sorting, without drug treatment.

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The fluorescent substrate, FDG (fluorescein di- $\beta$ -D-galactopyranoside, Molecular Probes, Inc.), staining was carried out using standard methods (see, e.g., Plovins et al., Appl. Environ. Microbiol. 60:4638, 1994). Cells were treated with trypsin to get a single cell suspension, counted, pelleted, washed twice with staining medium (10 mM Hepes and 4% fetal bovine serum in PBS), and resuspended in staining medium at  $10^7$  cells/mL. One hundred  $\mu$ L ( $10^6$  cells) were aliquoted into a 6 mL FACS tube (Falcon) and incubated in a 37°C water bath for 10 minutes. One hundred  $\mu$ L of 37°C pre-warmed 2 mM FDG (in sterile water) was added and mixed rapidly in the absence of direct light, then returned to the 37°C water bath for exactly one minute. FDG loading was stopped by adding 1 mL 4°C staining medium with a cold pipette. Cells were kept on ice and in the dark until FACS analysis. After the first round of FACS, rAAV/CMVlacZ-infected cells were enriched, but not pure, so a second round of FACS was carried out. Cells were grown-up in normal culture medium and prepared for FACS as described above, except that all cells, including the control cells, were treated with butyrate for 24 hours before FDG staining.

#### *Quantitative lacZ assay*

The assay was performed according to Lim et al. (BioTechniques 7(6):576, 1989), with slight modifications. HeLa cells were treated with trypsin and counted, washed twice with PBS, and resuspended in ice cold HBSS (Hank's Balanced Saline Solution, Sigma), to get  $2 \times 10^5$  cells/mL. One mL was aliquoted into a 15 mL conical tube and mixed gently with 1 mL of cold 2x ONPG (o-nitrophenyl- $\beta$ -D-galactopyranoside, Sigma) solution that contained 7 mM ONPG and 1% NP-40 (Nonidet P-40, Sigma) in HBSS. After incubation in a 37°C water bath for 30 minutes, the reaction was stopped by centrifugation at 3,500 rpm for 5 minutes. One mL of supernatant was removed for an OD reading at 420 nm.

*Cloning, PCR screening, and Southern blot analysis of rAAV/CMVlacZ-infected HeLa cells*

HeLa cells ( $1 \times 10^4$ ) were plated into one well of a 96-well plate, and pretreated with hydroxyurea, as described above. The cells were then infected with 100  $\mu$ L  
5 heat-inactivated viral lysate in a total volume of 250  $\mu$ L medium for 24 hours. The infection medium was then slowly and carefully aspirated, and 250  $\mu$ L fresh medium was added. Cells were grown-up and transferred from 96-well to 24-well plates, and then to 6-well plates when they became confluent. After 21 days of culture, rAAV/CMVlacZ-infected cells were cloned by limiting dilution. Briefly,  
10 the cells were treated with trypsin and counted, and then diluted in medium to a concentration of 5 cells/mL. One hundred  $\mu$ L was added to one well of a 96-well plate, and cultured for 21 days with medium changes every 3 or 4 days.

Single colony clones were treated with trypsin and half of the cells from each clone were transferred to one well of a 24-well plate; the other half was put into a  
15 eppendorf tube for DNA preparation. Cells in the eppendorf tubes were pelleted by centrifugation and the medium was aspirated. Forty five  $\mu$ L pure water was added to each tube and the samples were boiled for 10 minutes. When cooled, 5  $\mu$ L proteinase K (2 mg/mL in water) was added to each tube, which were then incubated at 56°C overnight. Proteinase digested samples were then boiled for 10  
20 minutes and chilled on ice. Two  $\mu$ L from each sample were used for one PCR reaction. The *lacZ* primers were 5'-GATGGTGCTGCGTTGGACTGAC-3' (nucleotides 1,844-1,865; SEQ ID NO:1) and 5'-TTCAACCACCGCACGATAGAGA-3' (nucleotides 2,228-2,207; SEQ ID NO:2), and PCR cycling conditions were: 92°C for 20 seconds, 57°C for 30 seconds, and  
25 68°C for 1 minute; for 40 cycles; and 68°C for 10 minutes.

PCR-positive clones were expanded gradually to 100 mm plates. A portion of the cells was plated into 12-well plates for drug post-treatment and X-Gal staining,

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as described above. Another portion of the cells was frozen, and another portion was used to prepare DNA for Southern blot analysis of integrated virus.

*Construction of rAAV vectors and production of recombinant virions*

The rAAV/CMVlacZ vector was constructed, and the viral lysate was prepared  
5 and titered as described (Luhovy et al., Biol. Blood Marrow Transplant. 2:24-30, 1996); the titer was  $1 \times 10^8$  per mL. The lysate was heated at 56°C for 15 minutes to inactivate wild type adenovirus.

The anti-sickling gene AS3 is similar to the anti-sickling gene AS2 described in McCune et al. (Proc. Natl. Acad. Sci. USA 91:9852-9856, 1994), but it contains an  
10 additional modification and has stronger anti-sickling effects. HS2 $\alpha\beta^{AS3}$  contains a 1.5 kilobase KpnI-BglII HS2 fragment, a -335  $\alpha$ -globin gene promoter, and a 2.4 kilobase NcoI-AvrII  $\beta$ -globin gene fragment. HS2 $\alpha\beta^{AS3}$  is subcloned into the SalI site of the rAAV cloning vector pMAV53. The rAAV/HS2 $\alpha\beta^{AS3}$  vector was then packaged in parallel with rAAV/CMVlacZ using the same conditions.

15 *Cloning, screening, and Southern blot analysis of rAAV/HS2 $\alpha\beta^{AS3}$ -infected K562 cells*

Cells ( $5 \times 10^3$ ) were infected with 50  $\mu$ L of heat-inactivated rAAV/HS2 $\alpha\beta^{AS3}$  lysate in a total volume of 200  $\mu$ L in a 96-well plate. After 24 hours of infection, cells were transferred to an Eppendorf tube, pelleted, washed twice with fresh  
20 medium, plated, and grown for 30 days. Cells were then cloned by limiting dilution as described above and analyzed by PCR and Southern blot hybridization.

*Reverse transcription (RT)-PCR analysis of rAAV/HS2 $\alpha\beta^{AS3}$ -transduced K562 clones*

Cells ( $7.5 \times 10^6$ ) from each clone were split into 3 wells of a 6-well plate.  
25 Medium (2x) containing sodium butyrate or trichostatin A was added at final

-22-

concentrations of 50 mM and 3  $\mu$ M, respectively. Normal medium was added to the third well as a control. After 24 hours, cells were collected by centrifugation, and total RNA was extracted with RNA STAT60 (Tel-Test, Friendswood, TX). Approximately 1  $\mu$ g of total RNA of each sample was reverse transcribed using the 1<sup>st</sup> Strand cDNA Synthesis Kit (Boehringer Mannheim), and 2  $\mu$ L (1/10 volume) of cDNA was used for PCR. A 25-cycle PCR (92°C for 30 seconds and 68°C for 90 seconds) was used to achieve linear amplification, and 0.5  $\mu$ L of [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol; 1 Ci = 37 Gbq; NEN) was included in each reaction. Three primers were used together to amplify both endogenous  $\alpha$ -globin cDNA and  $\alpha\beta^{AS3}$  cDNA. The forward primer (5'-ACTCTTCTGGTCCCCACAGA-3' (SEQ ID NO:3)) from the  $\alpha$ -globin 5' untranslated region was common to both endogenous  $\alpha$ -globin mRNA and  $\alpha\beta^{AS3}$  mRNA. One reverse primer (5'-GTTGGGCATGTCGTCCACGT-3' (SEQ ID NO:4)) was specific for endogenous  $\alpha$ -globin exon 2, and the other reverse primer (5'-TCACTAAAGGCACCGAGCAC-3' (SEQ ID NO:5)) was specific for  $\alpha\beta^{AS3}$  exon 2. PCR products (5  $\mu$ L) were mixed with 20  $\mu$ L of formamide loading dye, denatured at 70°C, and fractionated by electrophoresis on 5% denaturing polyacrylamide gels. The intensity of the PCR bands was quantitated on a PhosphorImager (Molecular Dynamics). The level of butyrate and trichostatin A induction of a  $\alpha\beta^{AS3}$  was calculated by the following formula: [counts per minute of  $\alpha\beta^{AS3}$  from butyrate or trichostatin A samples/counts per minute of  $\alpha$  from butyrate or trichostatin A samples]/[counts per minute of  $\alpha\beta^{AS3}$  from control samples/counts per minute of  $\alpha$  from control samples].

All publications referred to herein are incorporated by reference in their entirety. Other embodiments are in the following claims.



-23-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

(i) APPLICANT: THE UAB RESEARCH FOUNDATION

5 (ii) TITLE OF THE INVENTION: Use of Histone Deacetylase  
Inhibitors to Activate Transgene  
Expression

(iii) NUMBER OF SEQUENCES: 5

10 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Clark & Elbing LLP  
(B) STREET: 176 Federal Street  
(C) CITY: Boston  
(D) STATE: MA  
15 (E) COUNTRY: USA  
(F) ZIP: 02110

(v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
20 (D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER: PCT/US97/-----  
(B) FILING DATE: 13-JUN-97  
(C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/664,422  
(B) FILING DATE: 14-JUN-96  
(A) APPLICATION NUMBER: 08/---,---  
(B) FILING DATE: 27-MAY-97

30 (viii) ATTORNEY/AGENT INFORMATION:  
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(B) REGISTRATION NUMBER: 35,238  
(C) REFERENCE/DOCKET NUMBER: 04005/015W02

35 (ix) TELECOMMUNICATION INFORMATION:  
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(B) TELEFAX: 617-428-7045  
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

-24-

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATGGTGCTG CGTTGGAGTG AC  
22

- 10 (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
15 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTCAACCACC GCACGATAGA GA  
22

- 20 (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
25 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACTCTTCTGG TCCCCACAGA  
20

- 30 (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
35 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

-25-

GTTGGGCATG TCGTCCACGT

20

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

5

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCACTAAAGG CACCGAGCAC

20

What is claimed is:

-26-

## CLAIMS:

1. Use of a histone deacetylase inhibitor in the preparation of a medicament for increasing expression of a transgene in a cell.
2. A method for identifying an activator of transgene expression in a cell, said method comprising the steps of:
  - 5 providing a cell comprising a transgene;
  - culturing said cell in the presence of a candidate compound; and
  - monitoring expression of said transgene in said cell, wherein detection of an increased level of expression of said transgene in said cell cultured in the presence of said candidate compound, compared to the level of expression of
  - 10 said transgene in a control cell cultured in the absence of said candidate compound, indicates that said candidate compound is an activator of transgene expression.
3. The method of claim 2, wherein said compound is a histone deacetylase inhibitor.
- 15 4. The use of claim 1 or the method of claim 3, wherein said histone deacetylase inhibitor is a histone H3 or H4 inhibitor.
5. The use of claim 4 or the method of claim 4, wherein said histone H3 or H4 inhibitor is a derivative of butyrate, trichostatin A, or trapoxin.
6. The method of claim 2, wherein said transgene was introduced into said
- 20 cell using a viral vector.
7. The method of claim 6, wherein said viral vector is an adeno-associated viral vector.

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8. The method of claim 2, wherein said transgene is integrated into the genome of said cell.

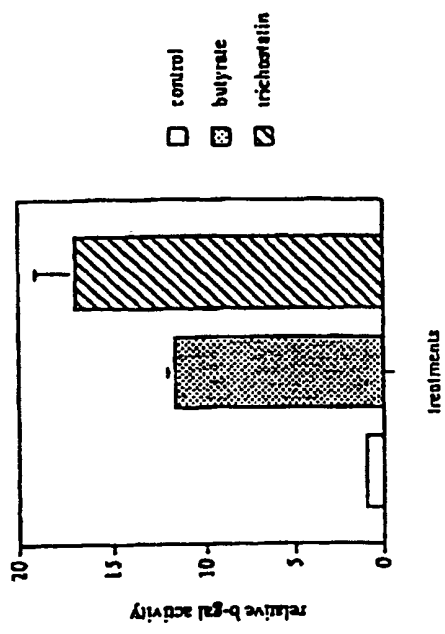
9. A cell comprising an integrated transgene that was introduced into said cell using an adeno-associated viral vector, in the absence of a selectable  
5 marker.

10. The method of claim 2 or the cell of claim 9, wherein said cell is a HeLa cell or a K562 cell.

11. The method of claim 2 or the cell of claim 9, wherein said transgene is the *lacZ* gene.

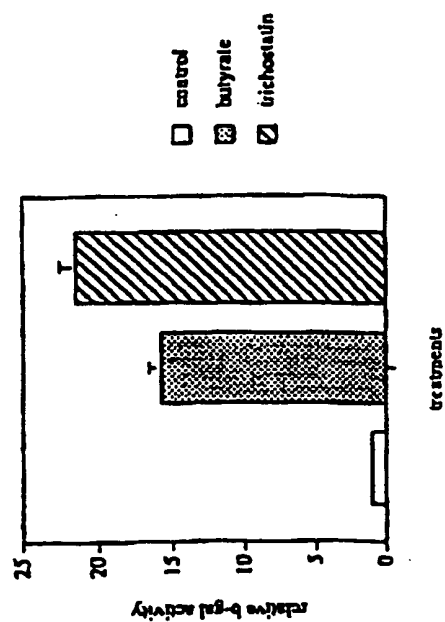
10 12. The method of claim 2 or the cell of claim 9, wherein said transgene is operably linked to a promoter.

13. The method of claim 12 or the cell of claim 12, wherein said promoter is the Cytomegalovirus promoter.



FACS sorted pool 2, without hydroxyurea pretreatment

FIG. 1B



FACS sorted pool 4, with hydroxyurea pretreatment

FIG. 1A

Fig. 2A

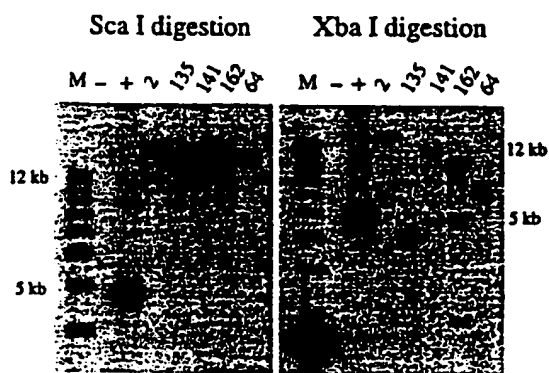
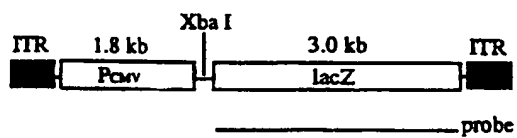


Fig. 2B

Fig. 2C



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Fig. 3

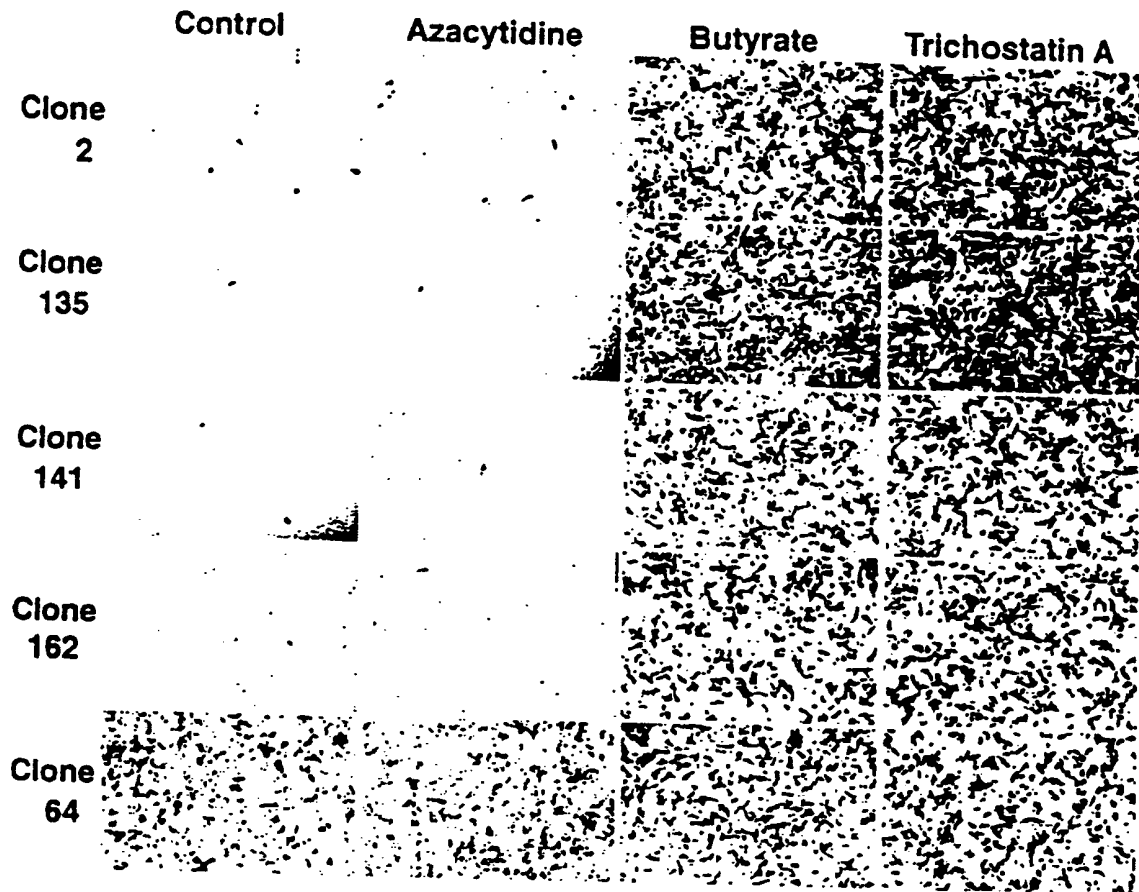




Fig. 4A

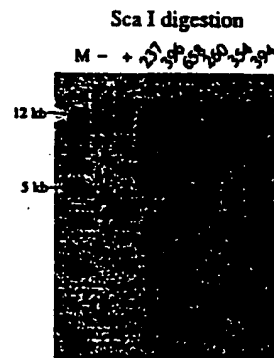


Fig. 4B

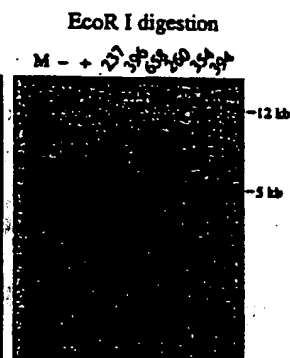


Fig. 4C

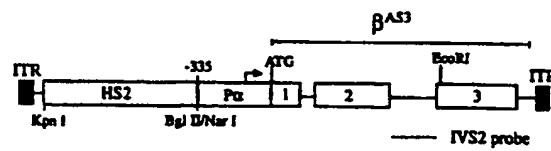
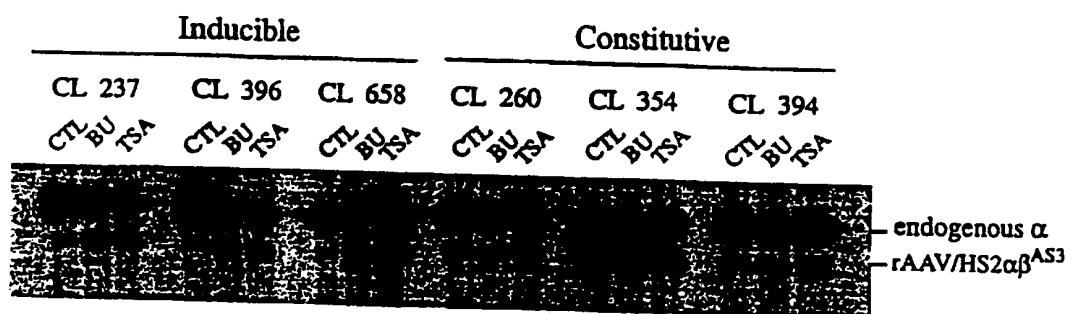
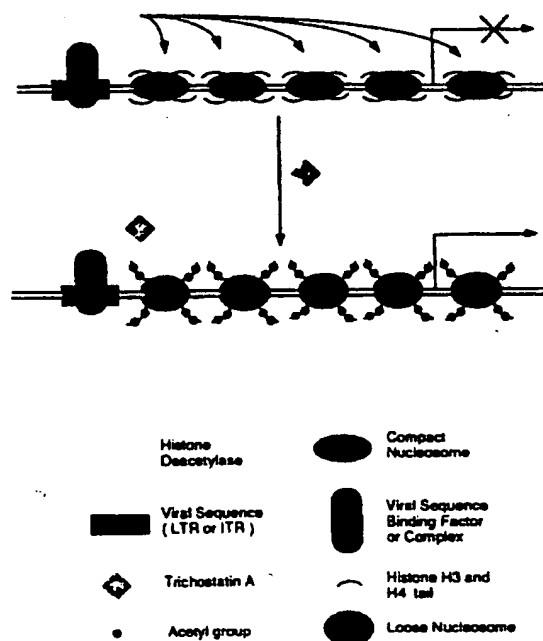


Fig. 5



6/6

Fig. 6



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/10262

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 31/70; C07H 21/04; C12N 15/86; C12P 21/02

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 70.1, 70.3, 172.1, 320.1, 375; 514/44; 536/23.1, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, WPIDS

search terms: histone deacetylase? inhibit? butyrate? trichostatin? trapoxin? transgene?

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	DORNER et al. Increased Synthesis of Secreted Proteins Induces Expression of Glucose-regulated Proteins in Butyrate-treated Chinese Hamster Ovary Cells. J. Biol. Chem. 1989. Vol. 264. No. 34, pages 20602-20607, especially the abstract.	1-4 ----- 5, 9-13
X ----- Y	GORMAN et al. Expression of Recombinant Plasmids in Mammalian Cells is Enhanced by Sodium Butyrate. Nucleic Acids Research. 1983. Vol. 11. No. 21. pages 7631-7648, especially the abstract.	1-4, 8 ----- 5, 9-13
X ----- Y	GOLDBERG et al. Elevation of Large-T Antigen Production by Sodium Butyrate Treatment of SV40-Transformed WI-38 Fibroblasts. J. Cell. Biol. 1992. Vol. 49. pages 74-81, especially the abstract.	1-4, 8 ----- 5, 9-13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

13 AUGUST 1997

Date of mailing of the international search report

09 SEP 1997

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/10262

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Whittaker et al. Secretion of Soluble Functional Insulin Receptors by Transfected NIH3T3 Cells. J. Biol. Chem. 1988. Vol. 263. No. 7. pages 3063-3066, especially the abstract.	1-4, 8 ----- 5, 9-13
X --- Y	GLAUBER et al. 5'-Flanking Sequences Mediate Butyrate Stimulation of Embryonic Globin Gene Expression in Adult Erythroid Cells. Mol. Cell. Biol. 1991. Vol. 11. No. 9. pages 4690-4697, especially the abstract and page 4691, col. 2, lines 9-23.	1-4, 6-7 ----- 5, 9-13
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**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US97/10262

**A. CLASSIFICATION OF SUBJECT MATTER:**

US CL :

435/6, 69.1, 70.1, 70.3, 172.1, 320.1, 375; 514/44; 536/23.1, 24.1